Analysis of synaptic protein networks by interaction proteomics

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Chapter 2

Identifying true protein complex constituents in interaction proteomics: The example of the DMXL2 protein complex

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Abstract

A typical high-sensitivity antibody affinity purification-mass spectrometry experiment easily identifies hundreds of protein interactors. However, most of these are non-valid resulting from multiple causes other than interaction with the bait protein. To discriminate true interactors from off-target recognition, we propose to differentially include an (peptide) antigen during the antibody incubation in the immuno-precipitation experiment. This contrasts the specific antibody–bait protein interactions, versus all other off-target protein interactions. To exemplify the power of the approach, we studied the DMXL2 interactome. From the initial six immunoprecipitations, we identified about 600 proteins. When filtering for interactors present in all anti-DMXL2 antibody immuno-precipitation experiments, absent in the bead controls, and competed off by the peptide antigen, this hit list is reduced to ten proteins, including known and novel interactors of DMXL2. Together, our approach enables the use of a wide range of available antibodies in large-scale protein interaction proteomics, while gaining specificity of the interactions.

Introduction

Interaction proteomics analyses are increasingly used in Neuroscience to delineate the constituents of signaling protein complexes (Li et al., 2010). The identification of novel protein interactions often serves as an entry point for subsequent functional studies aiming at elucidating the role of the interaction in the regulation of activity of the target protein, the complex, and / or of the corresponding pathway. Recent examples come from a series of reports on glutamate receptor associated proteins and ion channel associated proteins (Schwenk et al., 2009; von Engelhardt et al., 2010; Muller et al., 2010), from which the mechanistic aspects of a number of novel regulatory pathways underlying synaptic plasticity were discovered. Following these successes, large-scale analysis is now in progress to examine the synapse protein interactome (Li et al., 2010; Klemmer et al., 2009).

Interaction proteomics analysis generally relies on the use of affinity purification followed by mass spectrometry, which measures the direct and indirect binding of proteins to a defined target (Wodak et al., 2009; Pflieger et al., 2011). Affinity purification is often performed using antibodies raised to the (bait) protein of interest. To date, large collections of well-defined antibodies are available (e.g. from Neuromab, Epitomics, SYSY, Abgent, Genscript, Santa Cruz) that form a suitable starting point for interaction proteomics analysis. In a typical experiment, the tissue expressing the bait, usually a specific brain region, or more specifically a biochemically isolated subcellular organelle, is solubilized in an appropriate detergent. The antibody directed to the bait protein is then used to immuno-precipitate (IP) the bait and the associated proteins. Alternatively, tandem affinity purification tagging methodology with a gene targeting approach in mice has been reported for the isolation of a synapse protein complex (Fernandez et al., 2009). Whereas this technique is routinely applied in simple model systems such as yeast and cell cultures (Volkel et al., 2010), its application for protein interaction analysis in the mammalian brain is technically challenging, costly and time consuming, and therefore most likely will be applied to only a small number of selected proteins.

The immuno-isolated protein complex is commonly fractionated with SDS-PAGE, which separates proteins according to their mass. Proteins are then digested by trypsin, and the derived peptides are subjected to LC-MS-MS for protein identification (Chen et al., 2011). Given a current proteomics set-up and sensitivity of the mass spectrometer instruments, an IP-MS experiment will easily detect anywhere between 100 and 400 proteins (Malovannaya et al., 2010; Bildl et al., 2012). In most cases, only a small fraction of the identified proteins are true interactors of the bait protein. This high number of proteins comes from different sources. Typically they arise from (i) non-specific binding of proteins to the bead, (ii) non-specific binding of proteins to the antibody, (iii) sporadic appearance of proteins which have no affinity to the beads or the antibody but are captured by chance during IPs at generally low level, (iv) recognition of off-target proteins by the presence of irrelevant antibodies contained especially in the polyclonal antibodies preparations and (v) cross-reactivity of an antibody to different proteins containing the same antigenic site. To address these different binding modes, experiments with negative controls are often incorporated into the workflow. Inclusion of an empty bead control gives insight into (i) and a non-functional / pre-immunized serum antibody may help to shed light on (ii). The sporadic appearance of low-abundant proteins detected by MS (ii) may be counteracted by multiple replications. Generally,

these steps (i–iii) can discriminate a substantial number of unwanted interactions. The off-target recognition and cross-reactivity can be quite problematic. The use of knockout animals with protein deleted tissue as a reference is a good solution to this, i.e. proteins that are specifically present in the IP from wild-type littermate samples but not in knockout samples most likely represent true protein interactors (von Engelhardt et al., 2010). However, due to limited availability of knockout animals, this negative control is applicable only to specific cases. In particular, when aiming at large-scale proteomics interaction experiments, straightforward methods for dealing with 'non-specific' interactions need to be available. Recently, bioinformatics modeling of IP data have been proposed to distinguish interacting proteins from contaminants (Bildl et al., 2012; Choi et al., 2011; Lavallee-Adam et al., 2011).

Here, we investigated a simple method to distinguish true protein interactors from hundreds of proteins identified from an interaction proteomics experiment. We used two antibodies recognizing different epitopes to exclude the possible cross-reactivity (see point (v) above). We included (peptide) antigen during the antibody incubation step in an IP experiment to block the intended antibody–bait protein interaction in order to get insight into recognition of off-target antigenic sites (see point (iv) above). As a consequence, the bait protein interactors will be absent while all other proteins are retained. In case there is no off-target recognition, peptide blocking will serve as a control revealing proteins that bind non-specifically to the bead and / or antibody (points (i) and (ii)). Next, to recover potential false negatives, we compared the protein quantities between the controls and the IPs; true interactors should be significantly enriched in the IPs. Together, these approaches address most aspects of false recognition, and simplify the interpretation of the IP-MS data. To address this issue experimentally, we investigated this approach using the DMXL2 (Rabconnectin-3).

DMXL2 is highly expressed in the brain and forms tight interaction with WDR7 (Kawabe et al., 2003). This protein is reported to associate with synaptic vesicles at the synapse, and may serve as scaffold molecule for both Rab3 GEP and GAP on synaptic vesicles (Nagano et al., 2002). Recent studies on different mammalian cell types and insect tissue further implicated DMXL2 as regulator of Notch signaling via modulation of V-ATPase activity (Yan et al., 2009; Sethi et al., 2010). Whether this process also occurs in the brain is unclear.

In the present study, we designed an IP-MS protocol including the use of a blocking peptide, two antibodies, and large number of replicates (of controls) to examine the DMXL2 protein complex. We revealed WDR7, a known interactor of DMXL2, next to the vesicular proton pump, comprising of various V-ATPase subunits, and others, as novel DMXL2 interactors.

Materials and methods

Eight- to ten-week-old C57Bl6 mice were decapitated and the brain was immediately removed. The hippocampus was dissected on ice and directly frozen. The tissue was stored at –80 °C until further use.

Hippocampi were homogenised in ice-cold homogenization buffer (0.32 M sucrose, 5 mM HEPES, pH 7.4) containing protease inhibitor (Roche Applied Science, Indianapolis, IN, USA). Cell debris and nuclei were removed by 1000 × *g* centrifugation for 10 min. The supernatant was spun at 100,000 × *g* for 2 h to obtain a pellet enriched in synaptosomes and microsomes (P2 + microsome). Protein concentration was determined by Bradford assay and adjusted to 10 µg/µL, and 5 mg proteins were used for a single IP. The P2 + microsome fraction was extracted for 1 h at 4 °C in an equal volume of a buffer containing 2% Triton X-100, 150 mM NaCl and 25 mM HEPES, pH 7.4. After centrifugation for 20 min at 20,000 × *g*, supernatant was pipetted into an Eppendorf tube. The pellet was extracted once more with 1% Triton X-100 containing 150 mM NaCl, 25 mM HEPES, pH 7.4. The two supernatants were pooled and served as input material for the IP experiment. In a typical IP, the extract was incubated with 10 µg antibody overnight at 4 °C. To reveal the proteins associated with the beads, the extract was incubated with the beads (bead control). For a peptide-blocking experiment, the extract was incubated with 10 µg antibody in the presence of 20 µg peptide antigen. The two anti-DMXL2 antibodies were custom made by Genscript against the synthetic peptides CHEDGEREGSPRTHP and CISEDSTKKPQSYED. The V-ATPase Subunit A Antibody was catalog antibody from Genscript.

After incubation, 50 µL slurry of protein A/G PLUS Agarose beads (Santa Cruz, CA, USA) were added and incubated at 4 °C for 1 h. The beads were washed four times with HEPES buffer (25 mM HEPES, 150 mM NaCl, pH 7.4) containing 0.1% Triton-X100, mixed with 5 × SDS sample buffer and heated to 98 °C for 5 min. To block cysteine residues, 5 µL 30% acrylamide was added and incubated at room temperature for 30 min. Proteins were resolved on a 10% SDS polyacrylamide gel. After staining with Coomassie Blue, each sample lane was cut into five pieces, destained and the proteins digested with trypsin overnight at 37 °C. The tryptic peptides were dried, re-dissolved in 20 µL 0.1% acetic acid and subjected to LC-MS-MS analysis as previously described (Chen 2011).

MS-MS spectra were searched against an IPI mouse database (IPI, Mouse, V3.79) with the ProteinPilot™ software (version 3.0; Applied Biosystems, Foster City, CA, USA; MDS Sciex) using the Paragon algorithm (version 3.0.0.0) as the search engine. The search parameters were set to cysteine alkyation with acrylamide, and digestion with trypsin. The detected protein threshold [unused protscore (confidence)] in the software was set to 0.10 to achieve 20% confidence, and identified proteins were grouped to minimise redundancy. To examine the enrichment of proteins, we applied unpaired *t*-test of IP against peptide blocks and the bead controls, respectively.

Results and discussion

We carried out the IP experiments in three general conditions. (i) Typical IP experiments using an anti-DMXL2 antibody for the IP. (ii) Typical control experiments containing empty beads. (iii) New control experiments, in which peptide antigen and the anti-DMXL2 antibody were both present. Here, two antibodies raised against different DMXL2 epitopes were used in separate experiments.

In this experiment, we included the unused values generated from the software ProteinPilot as a semi-quantitative means for relative comparison (Klemmer et al., 2009). The 'unused' value is defined as a summation of protein scores from all the nonredundant peptides matched to a single protein. Peptides with confidence of >99 have a protein score of 2; >95 have a protein score of 1.3, >66 have a protein score of 0.47, etc.

In each IP experiment, around 200–300 proteins were identified. When the results of six IPs were merged, we identified a list of about 670 proteins with unused value >1.3 (see Supporting Information Table 1). Many of these proteins were sporadically present at generally low unused values across different IP or control experiments with no pattern in any particular condition. They likely represent the randomly occurring non-specific interactions due to sensitivity of the experiment. This argues that multiple IPs using the same antibody should be done to determine this type of background. There were also proteins abundantly present in most of the experiments, which may include those that bind to the beads.

To remove contaminants (proteins with origin external to the protein source) we deleted the proteins representing antibodies, trypsin and keratins. This reduced the list to about 560 proteins. We then excluded proteins that were present in both bead and peptide control experiments. We took into account that a protein stably interacting with DMXL2 should be found in all typical IP experiments (to address the problem of random background binding). The use of two antibodies further excluded the possibility of antibody cross-reactivity. Together, the number of potentially true interacting proteins was reduced to ten (Table 1). As expected, the proteins of nonspecific binding did not show any enrichment. The stringent criteria probably underestimate the number of DMXL2 interactors (see also below). We are also aware that proteins identified in IPs by a single antibody may still be valid interactors. Thus, independent biochemical approaches and / or reverse IPs will always be required for verification.

The DMXL2 interactors fall into several functional groups. WDR7 is a known DMXL2 interactor (Kawabe et al., 2003). Accordingly, this protein was identified with high unused value. WDR7 was reported to bind Rab3 GEP (Kawabe et al., 2003) and so the DMXL2-WRD7 complex may regulate neurotransmission via modulation of Rab3 activity on synaptic vesicles.

The vesicular V-ATPase has been implicated in DMXL2 functioning, in particular in the Notch signalling pathway (Yan et al., 2009; Sethi et al., 2010). We identified in this experiment a single subunit, the ATP6V1C1, suggesting the physical interaction of DMXL2 and V-ATPase. Obviously, a functional V-ATPase comprises many subunits, which are expected to be detected. A problem is that various V-ATPase subunits are routinely present in control experiments and therefore are often excluded from the list of the interactors. How to differentiate true interactors from non-specific binding? Table 2 lists all the V-ATPase subunits detected. The substantial enrichment of V-ATPase in

the IP experiments compared to the peptide block and the bead controls strongly implicates the interaction of DMXL2 to V-ATPase complex. We have performed reverse IP using an anti-ATP6V1A antibody. DMXL2 was identified with an unused value of 17.4, and was not detected in the peptide block and bead control experiments (Supporting Information Table 2), thereby validating the interaction of both proteins.

Other DMXL2 interactors include proteins involved in modulation of Actin dynamics, i.e. CYFIP2 and NCKAP1 belonging to the WAVE complex (Insall and Machesky, 2009). This suggests that DMXL2 may be involved in Actin-based processes, such as protein trafficking and regulation of cell / synapse morphology.

Taken together, we have demonstrated the importance of using a high number of replicates, both for IPs and controls, to exclude the random noise and to differentiate true interactors from those of non-specific binding by the use of peptide antigen block. The statistical analysis of enrichment of proteins in the IPs from a single antibody is shown in Supporting Information Table S1. In total, 62 proteins showed significant enrichment at $p < 0.01$, which include five V-ATPase subunits and six proteins from the CYFIP complex. Future work might make use of improved quantitation, including the consideration of the size of the protein, the spectral counting and the signal intensities from the MS and MS-MS spectra (Cox et al., 2011), for the global analysis of protein complexes. To this end, an algorithm is currently under development that incorporates these features, and will be applied for the analysis of data sets generated from large-scale analysis of interaction proteomics of synaptic proteins that is in progress in our laboratory.

Table 1. The DMXL2 interactors are blocked by peptide antigens.

Gene name	DMXL2 A	DMXL2 A	DMXL2 A	DMXL2 A	DMXL2 A Peptide block A	DMXL2 A	DMXL2 A Peptide block A	DMXL2 B	DMXL2 B Peptide block B
DMXL2	139.74	196.49	160.99	267.01	6.97	255.54		312.14	2.32
CYFIP2	48.19	42.17	9.64	40.48		40.66		6.77	
WDR7	45.05	30.12	35.37	45.39		25.61		55.28	
NCKAP1	28	29	18.8	30.11		20.85		3.59	
ATP6V1C1	8.7	14.44	15.17	20.37		14.73		11.76	
PFKL	13.02	4.36	8.85	4		13.34		15.67	
PRKCC	8.05	12.02	12.7	14.03		8.36		6.03	
PFKP	3.31	2.87	2.87	4		4.08		7.93	
MATR3	2	2	2.01	5.32		4		4	
IDH3G	2.92	2	2.04	3.4		2		11.74	
DYNC1H1	4.15	2.14	6.23	12.62		2		1.54	
GSTP1	27.33	27.3	22.55	52.23	72.21	56.02	55.92	16.27	31.4
ATP1A3	40.49	48.43	43.72	44.51	42.88	47.33	26.03	28.49	42.31
HSPA8	13.9	18.82	25.7	34.46	20.41	33.66	19.33	35.05	24.37
NAT13	7.51	13.8	8.33	10.98	20.75	18.92	17.38	13.67	14.72
SPNA2	7.52	11.3	7.9	8.9	24.55	5.76	16.69	4.42	25.82
GLUL	5.34	2.01	2.18	12.88	17.93	12.22	12.98	11.61	15.54

These proteins in the upper panel were present in all IPs and absent in all controls (except DMXL2, see also Supporting Information Table S1), whereas the non-specific binding proteins (lower panel, gene names italic) were equally present in peptide-blocking experiments. Data in this table highlight the power of using a blocking peptide control. DMXL2-A and DMXL2-B were IPs using two different anti-DMXL2 antibodies. Peptide blocks A and B were IPs in which the antibodies were co-incubated with their respective antigen peptides A and B. The values are the unused value given by the Proteinpilot algorithm. The low or absent unused values of DMXL2 in the peptide block experiments indicate the (near) complete abolishment of DMXL2-antibody interaction.

Table 2. The identified V-ATPase subunits showed general enrichment in the IPs.

Gene name	DMXL2 A	DMXL2 A	DMXL2 A	DMXL2 A	DMXL2 A Peptide block A	DMXL2 A	DMXL2 A Peptide block A	DMXL2 B	DMXL2 B Peptide block B	Con	Con	Con	Con	Con	Con	Con	Con	Con	Con
ATP6V0A1		18.6	24.3	14.2	1.7	19.4		10.1											
ATP6V0C						1.7													
ATP6V0D1	2.8	11.6	10.9	7.3		10		6.7	2.9							2			2
ATP6V1A	31.9	40.9	44.3	47.8	8.3	51.9	7.8	44.7	13.1						1.5	6.4		2	
ATP6V1B2	4.8	23.3	22.3	27.3	8	24.7	2	26									2	2	4.1
ATP6V1C1	8.7	14.4	15.2	20.4		14.7		11.8											
ATP6V1D	4.7	12.5	12.9	16.2		8.7	2	8.3											
ATP6V1E1	9.8	13.8	17.9	12		7.1		4.2											4
ATP6V1F						2.1													
ATP6V1H				2															

The V-ATPase subunits were identified at different levels because they differ in copy numbers and size; proteins with higher copy number and / or larger size were generally identified with higher unused values. The V0 domain consists of six copies of 17 kDa subunits (c) and a single copy of 100 kDa (a), and 38 kDa (d), subunits. The V1 is consists of three copies of 70 kDa (A) and 60 kDa (B) subunits and a single copy of 40 kDa (C), 34 kDa (D), 33 kDa (E), 14 kDa (F), and 50–57 kDa (H) subunits. DMXL2 A and DMXL2 B were IPs with two different anti-DMXL2 antibodies. Pept A and B were IPs in which the antibodies were co-incubated with their respective antigen peptides A and B. Con was the bead control without antibody.

Conclusion

Our study shows that IP experiments benefit from a series of specific controls including the use of large number of bead controls and exemplifies the use of the antigen-blocked differential screening. Specifically, we demonstrate the advantage of this method for the IP of the DMXL2, for which we can identify protein interactors even in the presence of considerable background. This procedure opens up the possibility of high-quality large-scale IP screening and subsequent mass spectrometry protein identification with the large collection of existing antibodies.

Supplementary data

The supplementary data of this manuscript can be found online:

http://onlinelibrary.wiley.com/store/10.1002/pmic.201100675/asset/supinfo/pmic7160-sup-0001-tables1.doc?v=1&s=0d2a11e503baa011ee3f82e50704e57ea13a4cfc

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